

POTENTIAL USE OF STEM CELL THERAPY FOR TRAUMATIC BRAIN INJURY

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ABSTRACT

Traumatic brain injury (TBI) accounts for a significant proportion of combat casualties as well as civilian trauma. Pharmaceutical interventions for brain trauma, in general, met with limited success and are often compounded by serious side effects. Recent research and technology on stem cell transplantation have opened up a novel means for CNS repair. This study aims to investigate the therapeutic potential of neural differentiation of stem cells from umbilical cord blood for neural repair following TBI. The present results have shown an *in vitro* differentiation of stem cells into neural lineages. These cells expressed neural markers nestin, Map2, GFAP and CNPase as detected by immunohistochemistry. Animals that received neural stem cells showed improved neurological functions when compared with control groups. This study has shown the differential and therapeutic potential of stem cells from umbilical cord blood which may be used as an alternative or a complementary therapy for traumatic brain injury.

1. INTRODUCTION

Traumatic brain injury is one of the leading causes of death and morbidity in young people. Pharmaceutical interventions for brain trauma, in general, have met with limited success and are often compounded by serious side effects. The adult brain is an organ that has a poor regenerative capacity after damage. Possible human interventions to rectify this situation include transplantation of neural stem cells (NSCs) into the damaged area.

Neural stem cells hold great promise as a source of new neurons or glial cells to the damaged central nervous system (CNS). NSCs are multipotential progenitor cells that have self-renewal activities and are capable of generating all three fundamental neural cell types (neurons, oligodendrocytes, and astrocytes) within the CNS. However, the number of NSCs in the adult brain is very limited and neurogenesis occurs only in two specific brain regions, the subventricular zone and hippocampal subgranular zone.

Recent *in vitro* analyses of human umbilical cord blood (UCB) have demonstrated the potential of UCB as a versatile and virtually unlimited donor source of neural stem cells for CNS repair. In comparison to adult peripheral blood, UCB displayed decreased immune responses to alloantigens. Cord blood banks now store large transplantable resources of UCB that have been analysed by immunological parameters. Cryopreserved UCB cells may fill the gap in finding a stem-cell transplant for patients.

UCB contains two main cell populations, the haematopoietic stem cells (HSCs) and the mesenchymal progenitor cells (MPCs). The presence of HSCs in UCB and their differentiation potential are well known, however those of MPCs have not been fully evaluated. MPCs are multipotent and are the precursors for marrow stroma, bone, cartilage, muscle and connective tissues.

In the present study, we explored the possibility of generating NSCs from MPCs. We postulated that MPCs in UCB could be induced to differentiate into NSCs, based on the observation that the majority of CD34-immunoreactive cells co-localized with S-100 protein, an intracellular calcium-binding protein present in a variety of glial cell populations as well as in Schwann's cell (Blumcke et al., 1999) and a small subpopulation was also immunoreactive for neuronal antigens.

2. MATERIALS & METHODS

2.1 Extraction of primary populations containing stem cells from human umbilical cord blood

Human umbilical cord blood was collected, with maternal consent, and approved by the Institutional Ethics Review Committee at Singapore General Hospital and the National Cancer Centre, Singapore.

Fresh mononuclear cells (MNCs) were obtained from diluted cord blood (1:2 diluted with PBS) by density centrifugation using Ficoll-Paque Plus (Amersham Pharmacia Biotech AB, Uppsala,

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Sweden). In brief, 35 ml of diluted UCB were carefully layered over 15 ml of Ficoll-paque. After centrifugation at 400g for 35 min at 20°C, MNCs were collected at the interphase, washed with PBS twice and ready for cell sorting.

2.2 Cell sorting

MPCs (CD34⁻ cell population) and HSCs (CD34⁺ cell population) were then enriched by a magnetic bead separation method using a Direct CD34 Progenitor Cell Isolation Kit (MACS; Miltenyi Biotec, Auburn, USA), following the manufacturer's protocol. MNCs were reacted with an antihuman CD34 mAb coupled with microbeads. The beadattached cells (CD34⁺) were separated on positive selection columns set in a magnetic field (Miltenyi Biotec). MPCs and HSCs were eluted and collected for culturing, respectively.

2.3 Cell differentiation

Isolated MPCs were pelleted by centrifugation and resuspended in NSC culture medium containing various growth factors and neurotrophic factors. Isolated HSCs were pelleted by centrifugation and resuspended in EPC culture medium containing various growth factors (e.g. vascular endothelial growth factor). The plates were incubated at 37°C in a humidified environment with 5% CO₂. Media were changed every 3 days.

2.4 Immunocytochemistry

The cultured neural stem cells were stained for proteins, which in normal brains are expressed primarily by neurons (nestin), oligodendrocyte (O4) and astrocyte (GFAP). In brief, cells were grown on coverslips and fixed with 2% paraformaldehyde. Nonspecific mAb binding was blocked by incubation with 1% sheep serum in 0.025% triton. Cells were then incubated with mouse monoclonal Nestin antibody or O4 antibody for 1 hr, washed in PBS-Tween and further incubated in sheep anti mouse FITC antibody for 30mins. Double- labelling was performed by further blocking the cells in 1% sheep serum followed by incubating the cells with rabbit polyclonal GFAP antibody for 1 hr. Sheep anti rabbit Cy3 antibody was then applied. After washing, cells were mounted on slides using a fluorescence mounting medium containing DAPI for nuclear staining. The slides were examined under a fluorescence microscope (Axiovert 25, ZEISS).

2.5 Fluorescent pre-labelling of stem cell-enriched populations

A Vybrant CFDA SE Cell Tracer Kit (V-12883, Molecular Probes) was used to label the cells. The cells were labeled by diluting the CFDA SE stock (10 mM in DMSO) into the cell suspension at the final concentration of 25 µM and incubated for 15 min at 37°C. After labeling, DMEM supplemented with 5% FCS was added to stop the labeling reaction, and cells were centrifuged and washed with PBS twice. These labeled cells were further incubated with their appropriate medium at 37°C overnight to allow excessive CFDA SE to diffuse out from them, preventing possible undesirable labeling of surrounding cells after transplantation by unbound CFDA SE. Once completed, fluorescent pre-labeled cells were ready for *in vivo* transplantation.

2.6 Lateral fluid-percussion brain injury

Male Sprague Dawley rats (250 ± 25 gm) were anesthetized and placed in a stereotaxic frame. The scalp and temporal muscle were reflected and a hollow female Luer-Loc fitting (4.5 mm) was fixed rigidly with dental cement to the animal's skull through a craniotomy centered over the left parietal cortex, 5 mm from the lambda and 4 mm from the sagittal suture with the dura left intact. The lateral fluid percussion model was applied to induce brain injury, in which brief displacement and deformation of brain was resulted from the rapid epidural injection of saline into the closed cranial cavity. Animals were subjected to 2.6 atm pressure pulses, which produced severe tissue damage in ipsilateral cerebral cortex and hippocampus. Sham-operated animals received anesthesia and surgery but were not subjected to trauma.

2.7 Transplantation

After pre-labeling with fluorescent probes, the adherent cells were collected in a suspension by treating with 0.25% (w/v) Trypsin–0.03% (w/v) EDTA solution. A sample of the cell suspension was taken for assessment of cell viability using trypan blue exclusion method. Each cell suspension contains 50,000 viable cells/µl in PBS was prepared. The suspension was then intravenously injected into the left external jugular vein (100 µl in 0.1 ml saline; i.e. 5,000,000 viable cells per rat) 72 hours after the brain injury. The saline-control animal was intravenously injected with 100 µl saline.

2.8 Neurological and behavioral tests

a. Acoustic startle response

The animal was placed in the acoustic startle apparatus (San Diego, USA) compartment and was

first acclimatized for three minutes in the presence of 70 dB of broad-band white noise. Following this period, the animal was immediately exposed to fifty 50-millisecond bursts of white noise at 120 dB with a five-second period of 70 dB noise between each burst. The peak amplitude of each startle reflex movement was recorded using the SR-lab software (San Diego, USA). Acoustic startle response was carried out before TBI and 1 week after cell transplantation.

b. Fore Limb Grip-Strength Test

Forelimb grip strength was determined using a grip strength meter (Columbus Instruments, Ohio, USA). The animals were placed on the electronic digital force gauge that measures the peak force exerted on it by the action of the animal. While drawing along a straight line leading away from the sensor, the animal released at some point and the maximum force attained was stored on the display. The highest reading (in Newtons) of three successive trials was taken from each animal. The animals were subjected to grip strength test before TBI and 1 week after cell transplantation.

3. RESULTS

3.1 Stem cell culture

After induction, the cultured cells displayed some external features of neurons or glia (Fig. 1). The cells were immunoreactive for neuronal-lineage specific marker nestin (Fig. 2), Oligodendrocyte marker, O4 and astrocyte marker, GFAP (Fig. 3).

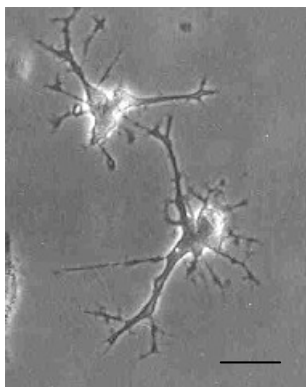


Figure 1: Light micrograph showing neural-like cells with processes. Bar=20 μ m.

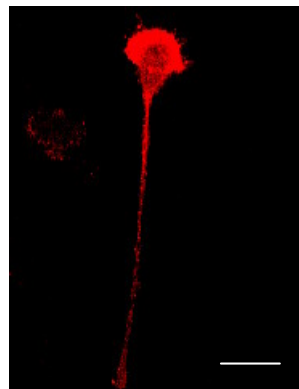
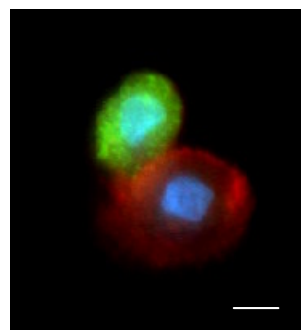


Figure 2: Immunohistochemical staining showing a cultured cell expresses marker for neural stem cell, nestin. Bar=20 μ m.



Figures 3: O4 & GFAP double immunofluorescence showing differentiation of CD34- cells into neural stem cells *in vitro*. Cultured cells stained with O4 (green), GFAP (red) and DAPI (blue). Bar=5 μ m.

3.2 Biodistribution of the transplanted cells

The transplanted cells appeared to survive and exhibited homing specificity. Fluorescent CFDA SE pre-labeled cells injected intravenously were localized exclusively in the injured cerebral cortex at 1 day after transplantation (Fig. 4). Fluorescent pre-labeled cells were undetected in the contralateral cerebral cortex. In the vital organs (e.g. spleen, liver, kidney, lung and heart) obtained, hardly any fluorescent pre-labeled cells were observed.

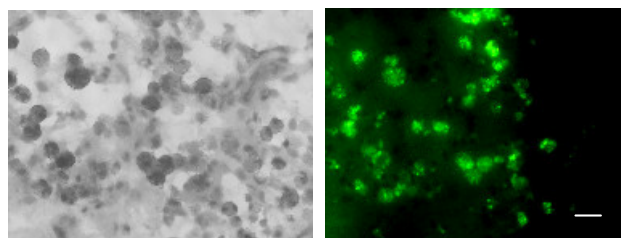


Figure 4: (a) Light micrograph and (b) fluorescence micrograph showing transplanted cells (green fluorescent cells) survived and migrated into the ipsilateral injury site. Bar= 10 μ m (for Fig a, b).

3.3 Functional outcomes

One week after transplantation, neurological deficits were observed in rats subjected to TBI in the acoustic response (878.3 ± 50.1 vs 344.3 ± 78 ; sham vs TBI+saline; $P < 0.05$) (Fig. 5). However, TBI-induced neurological deficits were significantly ameliorated in rats that received cell transplants when compared with those receiving saline injection (672.1 ± 37 vs 344.3 ± 78 ; TBI+UCBs vs TBI+saline; $P < 0.05$) (Fig. 5). UCBs induced functional recovery, however, did not reach levels comparable to intact sham-operated animals (878.3 ± 50.1 vs 672.1 ± 37 ; Normal vs TBI+UCBs $P < 0.05$).

Similarly, significant difference was observed in grip strength test between TBI and normal rats ($10.77 \pm 0.18 \text{ Nm}^2$ vs $9.18 \pm 1.16 \text{ Nm}^2$; sham vs TBI+saline; $P < 0.05$) (Fig. 6). Rats with UCB transplants exhibited significantly improved grip strength scores compared with saline-control 1 week postgrafting ($9.18 \pm 1.16 \text{ Nm}^2$ vs $11.13 \pm 0.25 \text{ Nm}^2$; TBI+saline vs TBI+UCBs) (Fig. 6).

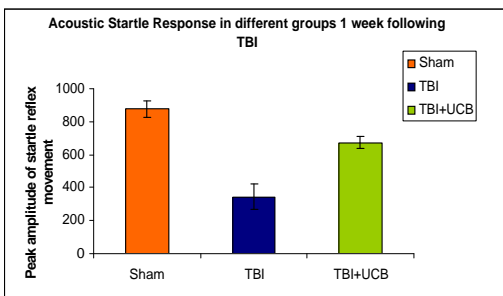


Figure 5: An improved acoustic startle response is observed in rats subjected to TBI with injected cells when compared with rats with saline injection. (*denotes significant difference from TBI+saline. $p < 0.05$)

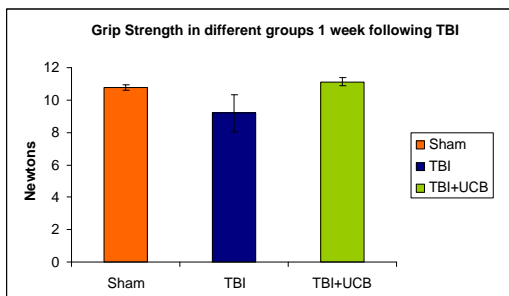


Figure 6: Grip strength test shows an improved function in traumatic rats with injected cells when compared with rats given saline injection. (*denotes significant difference from TBI+saline. $p < 0.05$)

4. DISCUSSION

In the present study, some cultured cells displayed some neuronal-like morphology and expressed nestin, a marker for neural stem cells as detected by immunocytochemistry. Some cultured UCB cells also expressed O4, a marker for oligodendrocyte and GFAP, a marker for astrocyte. These data provide evidence that CD34⁺ cells are capable of differentiating into cells that express several neural proteins and resemble neurons. In this connection, *in vitro* differentiation of MSCs in human umbilical cord blood into neuron-like cells has been reported by Hou et al., 2003. In addition, recent study by Jang et al (2004) has also shown that human cord blood-derived CD133⁺ hematopoietic stem cells could trans-differentiate into neural cell types of neuron-like cells, astrocytes, and oligodendrocytes by RA treatment.

Transplanted cells also have the capacity to migrate extensively and show “homing” specificity. In the present study, transplanted cells have been shown to migrate through the blood-brain barrier into the region of injured cerebral cortex of rats following TBI. In our previous study, we have also demonstrated migration of bone marrow stem cells into the injured cerebral cortex using the same animal model (manuscript has been submitted to Stem Cells).

A major finding in this study is that the TBI rats have shown improvement in neurological functions after UCBs transplantation. It has been reported that transplantation of UCBs ameliorated neurological deficits induced by brain ischemia (Vendrame et al., 2004; Willing et al., 2003). It is suggested that the functional improvement in the present TBI rats may have been mediated by neurotrophic or neuroprotective factors secreted from the surviving transplanted cells, which could have upregulated host brain plasticity in response to TBI. Numerous studies suggest that neurotrophic factors play important roles in brain plasticity. Neurotrophic factors can enhance neuronal sprouting (Cohen-Cory & Fraser, 1995), synaptogenesis (Arvanian et al., 2003), long-term potentiation (Nawa H et al., 1997), and neurotransmission (Kang & Schuman, 1995) and increase neurotransmitter release (Knipper et al., 1994). It is speculated that the grafted cells and/or the host brain cells presumably would be stimulated by the grafts may likewise produce neurotrophic factors and participate in functional reorganization in the host brain.

In summary, present results demonstrate that neural stem cells can be derived from mesenchymal progenitor cells in umbilical cord blood. This study

has shown the differentiation potential of MPCs and demonstrates that UCB is a promising resource for future neuronal cell repair.

transmission in the adult hippocampus. *Science* 267: 1658–1662.

5. REFERENCES

Blumcke I, Giencke K, Wardelmann E, Beyenburg S, Kral T, Sarioglu N, Pietsch T, Wolf HK, Schramm J, Elger CE, Wiestler OD. The CD34 epitope is expressed in neoplastic and malformative lesions associated with chronic, focal epilepsies. *Acta Neuropathol (Berl)*. 1999 May;97(5):481-90.

Jang YK, Park JJ, Lee MC, Yoon BH, Yang YS, Yang SE, Kim SU. Retinoic acid-mediated induction of neurons and glial cells from human umbilical cord-derived hematopoietic stem cells. *J Neurosci Res*. 2004 Feb 15;75(4):573-84.

Hou L, Cao H, Wang D, Wei G, Bai C, Zhang Y, Pei X. Induction of umbilical cord blood mesenchymal stem cells into neuron-like cells in vitro. *Int J Hematol*. 2003 Oct;78(3):256-61.

Vendrame M, Cassady J, Newcomb J, Butler T, Pennypacker KR, Zigova T, Davis Sanberg C, Sanberg PR, Willing AE. Infusion of human umbilical cord blood cells in a rat model of stroke dose-dependently rescues behavioral deficits and reduces infarct volume. *Stroke*. 2004 Oct;35(10):2390-5. Epub 2004 Aug 19.

Willing AE, Vendrame M, Mallery J, Cassady CJ, Davis CD, Sanchez-Ramos J, Sanberg PR. Mobilized peripheral blood cells administered intravenously produce functional recovery in stroke. *Cell Transplant*. 2003;12(4):449-54.

Cohen-Cory S, Fraser SE (1995) Effects of brain-derived neurotrophic factor on optic axon branching and remodelling *in vivo*. *Nature* 378: 192–196.

Arvanian VL, Horner PJ, Gage FH, Mendell LM (2003) Chronic neurotrophin-3 strengthens synaptic connections to motoneurons in the neonatal rat. *J. Neurosci.* 23: 8706-12.

Nawa H, Saito M, Nagano T (1997) Neurotrophic factors in brain synaptic plasticity. *Crit Rev Neurobiol*. 11: 91-100.

Kang H., Schuman EM (1995) Long-lasting neurotrophin-induced enhancement of synaptic

Knipper M, da Penha Berzaghi M, Blochl A, Breer H, Thoenen H, Lindholm D (1994a) Positive feedback between acetylcholine and the neurotrophins nerve growth factor and brain-derived neurotrophic factor in the rat hippocampus. *Eur. J. Neurosci*. 6: 668–671.